THE INFLUENCE OF *PSEUDOMONAS AERUGINOSA* OUTER MEMBRANE ANTIGENS ON EXPERIMENTAL BONE INFECTION IN RABBITS

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ABSTRACT

To study the influence of *Pseudomonas aeruginosa* outer membrane antigens (POMAgs) on experimental osteomyelitis induced by this bacterium, forty four rabbits were divided into 3 groups. The 1st group inoculated intramedullary in the right tibia bone by 0.1 *p. aeruginosa* suspension (2×10^8 cfu/ml), the 2nd group immunized with POMAgs 0.4 ml (2.7 mg/ml protein concentration) subcutaneously, two doses with 2 weeks intervals, and inoculated intramedullary in the right tibia bone as in the 1st group. The third group served as negative control group. At 28 day post immunization the 2nd group showed significant increase in skin thickness (Delayed type hypersensitivity test –DTH-), and in day 30 post immunization, the immunized group showed significant increase in the level of IFN- and IgG compared with negative control group. At 30 days post inoculation with *P. aeruginosa* the white blood cells differential count showed a significant decrease in neutrophils and significant increase in basophils of both the 1st and 2nd groups compare with negative control group, also increase in mononuclear cells (lymphocytes and monocytes) in the 1st group only. The pathological lesions in the tibia bone in the 1st group was very severe, grossly the lesion extended from bone diaphysis to epiphysis and joint, while less severe gross lesion showed in the immunized group. The microscopic lesion in the 1st group at 5, 25 and 30 day post challenge showed a destructive change and no signs of healing, contrariwise the microscopic lesion in the 2nd group which was less severe and showed signs of healing signs. In conclusion results showed that immunization with POMAgs enhances both cellular (DTH and IFN-), and humoral immunity (IgG) and white blood cells differential count, also it enhance the inflammatory reaction against *P. aeruginosa* and give good protection to the bone.

KEYWORDS: outer membrane, *P. aeruginosa*, tibia, IFN- , IgG.

INTRODUCTION

Osteomyelitis is inflammation of the bone and bone marrow that is generally of a bacterial origin (Wu and Yuan, 2012). However, the commonest infecting organisms isolated from osteomyelitis were *Staphylococcus* (54%) followed by enterobacteriaceae (23%) that included [*proteus* spp (12.5%), *E.coli* (8%), *Klebseilla* (2.5%)], *P. aeruginosa* (18%), anaerobes (2.5%) and miscellaneous (2.5%) (Qureshi *et al*., 2009). In Mosul-Iraq the commonest microorganisms detected in osteomyelitis were *S. aureus* (29.3%), followed by *Pseudomonas* (18.5%), *E. coli* (15.8%), *S. epidermidis* (14%), *Proteus* (9.9%), and other microorganisms (12.6%) (Al-Habib and Aljumaily, 2010). *P. aeruginosa* produced a wide array of a complex virulence factors which make it difficult to determined which microbial factors needed to develop an effective vaccine that can stimulate a good immune response (Priebe and Pier, 2003). However, several *P. aeruginosa* vaccines were developed such as outer membrane proteins (Gocke *et al*., 2003, Thomas et al., 2003), cytosolic protein (Thomas *et al*., 2002), extracellular proteins, such as those of flagella (Doring and Dorner, 1997) and pili (Cachia *et al*., 1998), and extracellular polysaccharides, such as alginate (Theilacker *et al*., 2003) and lypopolysaccharides (LPS) (Pier, 2003). However the current study try to determined the role of *P. aeruginosa* in osteomyelitis and the protective effects of POMAgs against *P. aeruginosa* bone infection .

MATERIALS & METHODS

The bacterial isolate

P. aeruginosa obtained from the department of Biology/ college of Science/University of Baghdad. This bacteria was isolated from a patient with hospital contaminated burn wound. The diagnosis of this bacterial strain was made by the Central Public Health Laboratories/Ministry of Health/ Baghdad-Iraq.

Antigen preparation

Pseudomonas aeruginosa outer membrane was prepared according to the method of Mizuno and kageyama, 1987. The total protein concentration was measured according to Procedure of Bradford, 1976 and it estimated to be 2.7 mg/ml.

Experimental design

Forty four male local breed rabbits, weight range 1.5-1.75 kg were randomly divided into 3 groups, as following: the 1st group (n=18) inoculated intramedullary in the right tibia bone with 0.1 ml (2×10^8 cfu/ml) of *P. aeruginosa* suspension and served as positive control group, the 2nd group (n=18) was immunized with 0.4 ml of POMAgs (2.7 mg/ml protein concentration) subcutaneously, two doses with 2 weeks intervals, at 28 day post immunization this group subjected to delayed type hypersensitivity (DTH) test, and at 30 days post immunization rabbits inoculated intramedullary in the right tibia bone as in 1st group, the 3rd group (n=8): served as negative control group. Rabbits from both the 1st and 2nd groups were

sacrificed after 5 (n=6), 15 (n=6) and 30 (n=6) days post inoculation.

Surgical operation and challenge dose

The surgical operation and challenge dose (0.1 ml of bacterial suspension 2×10^8 cfu/ml) used in this experiment depend on the procedure of Kanellakopoulou *et al.* (2008) with little modification. Briefly, sedation of animals was made by intramuscular injection of 40 mg/kg of ketamine and 5 mg/kg of xylazine, then skin was incised about 2-3 cm along the shaft of the right tibia bone. A hole was made in the bone using a local modified sterile drill (2 mm drill bit), then 0.1 ml of bone marrow was aspirated and 0.1 ml of the prepared bacterial suspension (2×10^8 cfu/ml) was instilled using a 1 ml syringe, finally, the hole was plugged with sterile bone wax and the skin incision was closed by a silk suture (size 3-0).

Immunological examination

Delayed type hypersensitivity (DTH) test for cellular immunity was performed at day 28 post immunization and the thickness of skin measured after 24 hr and 48 hr.

Blood samples were collected directly from the heart at day 30 post immunization for serum interferon gamma (IFN-) assay using IFN- ELIZA kit, provided by Cusabio Biotech. CO., LTD. China, and serum immunoglobulin G (IgG) assay using rabbit IgG ELIZA kit, (Immunoperoxidase assay for determination of IgG in rabbit sera) provided by Immunology Consultants laboratory, Inc. Portland, USA.

White blood cell differential count (WBCsDC)

Blood samples were collected from 6 rabbits of each group at 30 days post inoculation to measure WBCsDC (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Blood smears stained with Wright stain and examined under the light microscope by counting 100 different cells on each slides and calculate the mean of 6 stained slides for each group.

PATHOLOGICAL EXAMINATION

Gross Examination

After scarification, the gross lesions were recorded and classified according to the grading of Rissing (Spagnolo *et al.*, 1993) as following: 0, absence of abscesses, sequestra, active bone formation, and erythema, 1, Minimal erythema, without abscesses or new bone formation, 2, erythema, with widening of the head and shaft and new bone formation, 3, abscesses, with new bone formation, sinus tract drainage, or grossly purulent exudates, 4, Severe bone resorption, abscesses, and diaphyseal or total tibial involvement.

Microscopic lesion

Bone specimens decalcified using formic acid then fixed in 10% buffer formaldehyde solution for 72 hr, then used the routine tissue section for preparation and staining with Hematoxiln and Eosin stain (Presnell and Schreibman, 1997).

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to analyzed the effect difference factors in study parameters. Least significant difference (LSD) test was used to significant compare between means in this study.

RESULTS & DISCUSSION

After 24 hr post examination, the skin thickness (Table 1) of the immunized group was increased in a mean value (1.839 ± 0.27), while after 48 hrs the mean value of skin thickness was (1.679 ± 0.24).

TABLE 1: Skin test values for immunized group

Time (period)	No.	Mean ± SE of Skin test values
24 hrs - zero h	7	1.839 ± 0.27
48 hrs- zero h	7	1.679 ± 0.24
LSD value	---	0.783 NS
P-value	---	0.646
NS: Non-significant.		

The results showed that the mean values of serum IFN- , 30 day post immunization, was significantly high (P<0.01).in the immunized rabbits (2nd group) (574.17 ± 30.34) compared with 3rd group (negative control group) (375.21 ± 58.87) with LSD value (135.915).

TABLE 2: value of serum IFN- .

The group	No.	Mean ± SE of IFN-
Outer membrane	6	574.17 ± 30.34 a
Negative control	6	77.07 ± 11.09 b
LSD value	---	135.915 **
P-value	---	0.0001
** (P<0.01).		
Means with different letters in same column differed significantly		

The mean values of serum IgG, 30 day post immunization, showed a significant increase (P<0.05) in the immunized rabbits (2nd group) (21.416 ± 1.99) compared with the 3rd group (negative control group) (15.747 ± 0.84) with LSD (4.827).

TABLE 3: value of IgG in serum

The group	No.	Mean ± SE of IgG
Outer membrane	6	21.416 ± 1.99 a
Negative control	6	15.747 ± 0.84 b
LSD value	---	4.827 *
P-value	---	0.0289
* (P<0.05).		
Means with different letters in same column differed significantly		

The results indicated that POMAgs stimulated cell mediated immune response in immunized rabbits, since DTH reaction is one arm of effector cell mediated immune response which mediated by CD4+ and CD8+ T cell cytokines production (Kobayashi *et al.*, 2001). POMAgs may stimulate the production of IFN- by Th1 cells, IFN- is a potent stimulator for recruitment of immune cells especially macrophages to antigen inoculation site leading to skin indurations (Dietert *et al.*, 2010).

Redness and thickness of the skin at POMAgs injection site may correlated to the blood vessels congestion and edema, in addition to immune cells accumulation due to lymphokines produced by Th1 subset which stimulated macrophages to produce monokines that facilitate vasodilation, increase blood vessels permeability, extravasation and chemoattraction of immune cells to the dermis and subcutaneous tissue inducing indurations and swelling at the site of inoculation during 24-48 hrs post exposure to antigen (Scott, 1993).

In other hand, the significant increase (P<0.01) of IFN- in immunized rabbits may suggested that antigen presenting cells (APCs) engulf and destroy POMAgs and presenting them by the major histochompatible II (MHC II), APCs and natural killer (NK) cell produce proinflammatory cytokines (IL-12, IFN- and nitric oxide) which are important to control intracellular pathogen (Ohteki and Koyasu, 2001).

POMAgs enhanced the production of serum IgG in the immunized group and this may be due to CD_4 T cells, which play an important role in immune protection, it divided into two groups, Th1 subset which is critical against intracellular microorganisms through the production of IFN- that enhance the isotype switching to IgG2a by T-dependent antibody response and Th2 subset which is critical against extracellular pathogens through the production of IL-4 that enhanced the isotype switch to IgE and IgG4 (Zhu and Paul, 2008).

Table 4 summarized the results of WBCsDC in which positive control group showed a significant decrease (P<0.05) in neutrophils and monocytes and significant increase in lymphocytes and basophils, while rabbits immunized with POMAgs showed a significant decrease in neutrophils and significant increase in basophils compared with negative control group, while eosinophils in both groups show no significant difference.

TABLE 4. White blood cells differential count

The group	Mean ± SE of blood count				
	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
Positive control	11.83 ± 0.79 b	81.00 ± 6.32 a	4.17 ± 0.27 b	0.67 ± 0.03 a	2.33 ± 0.04 a
Outer membrane	13.33 ± 0.85 b	76.00 ± 5.76 ab	7.67 ± 0.52 a	0.50 ± 0.02 a	2.50 ± 0.02 a
Negative control	19.17 ± 1.05 a	72.67 ± 5.31 b	7.17 ± 0.39 a	0.67 ± 0.03 a	0.333 ± 0.02 b
LSD value	4.271 *	8.377 *	2.053 *	0.348 NS	1.372 *
P-value	0.0294	0.0362	0.0385	0.268	0.0394
* (P<0.05).					
Means with different letters in same column differed significantly					

Previous study (McConnell *et al*., 2010) showed that high dose of *P. aeruginosa* lead to marked increase in circulating neutrophils and decrease in lymphocytes in a mice model of pneumonia during few hours, and these values may return to normal after 3 days, while (Chevaleyre *et al*., 2016) mention similar result in acute pneumonia pigs model, the current results contraindicate these studies regardless of the time duration of the experiment and animal models of these experiments. Further studies needed to confirm and explain these results.

Pathological examination
Gross lesions
At 5 day post inoculation the lesion in the non-immunized infected rabbits showed gross evidence of osteomyelitis which become more obvious at 15 day and the lesion characterized by discoloration, widening of the head and shaft of the bone, and replacement of smooth, shiny periosteum by a coarse dull surface. At day 30 post inoculation the lesion extend from diaphysis to epiphysis, new bone formation connected between fibula and tibia, the tibia become fragile with severe deformity represented by shortness and fusion with calcaneus bone and the whole ankle joint covered by new bone which lead to joint sclerosis (Figure 1). The gross lesions in the bone of immunized rabbits appeared at day 15 a post challenge and characterized by irregular elevated periosteum especially on the dorsal part of the bone, at 30 day the lesion still localized and did not extended to the ankle joint and it is limited to the lower part of the diaphysis in addition there is a small exostosis just below the surgical hole which covered with very light callus (Figure. 2). According to the above observations, the lesion can be classified in grade 4 for non-immunized group and grade 2 in the immunized rabbits according to the grading of Rissing (Spagnolo *et al.*, 1993).

FIGURE 1: Tibia bone (a), normal tibia from control negative group. (b), tibia form *P. aeruginosa* infected group 30 day post inoculation showed severe deformities including shortening of tibia and sclerosis of the ankle joint.

FIGURE 2: Tibia form immunized rabbit 30 day post infection showed irregular elevated periosteum with very light callus covered the surgical hole.

Microscopic lesion

In the non-immunized rabbit at 5 day post challenge the lesion characterized by hypercellularity of hemopoietic tissue and neutrophils infiltration in the bone marrow (Fig. 3), also aggregation of few neutrophils in the Haversian canals.
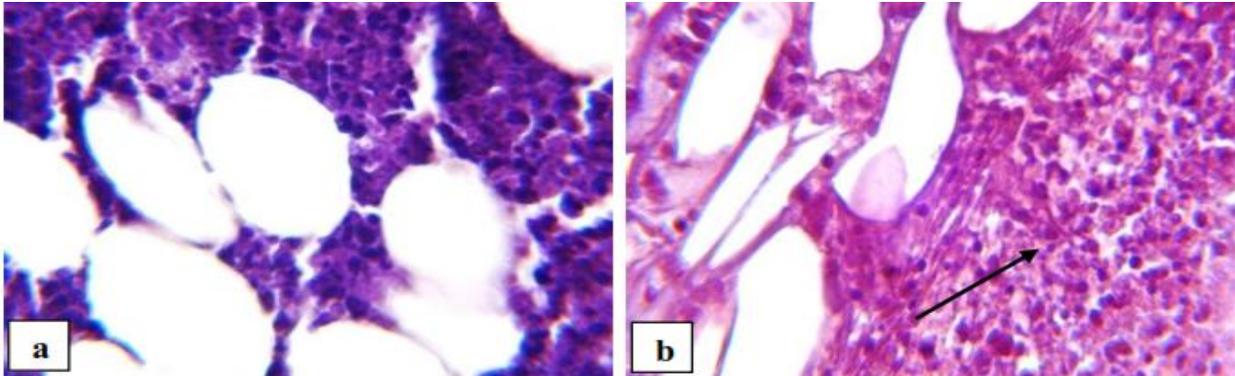
FIGURE 3: Tibia of non-immunized group at 5 day post challengen (H & E stain). (a) hypercellularity of hemopoietic tissues (×40), (b) neutrophils (arrow) infiltration in the bone marrow (×40).

At day 15 post infection the previous lesions become more severe and there is infiltration of neutrophils and F.C.T in the bone marrow, coagulative necrosis and loss of differential staining of marrow elements with circular space (pooled lipid), and neutrophils seen in the dilated Haversian canals (Figure 4).
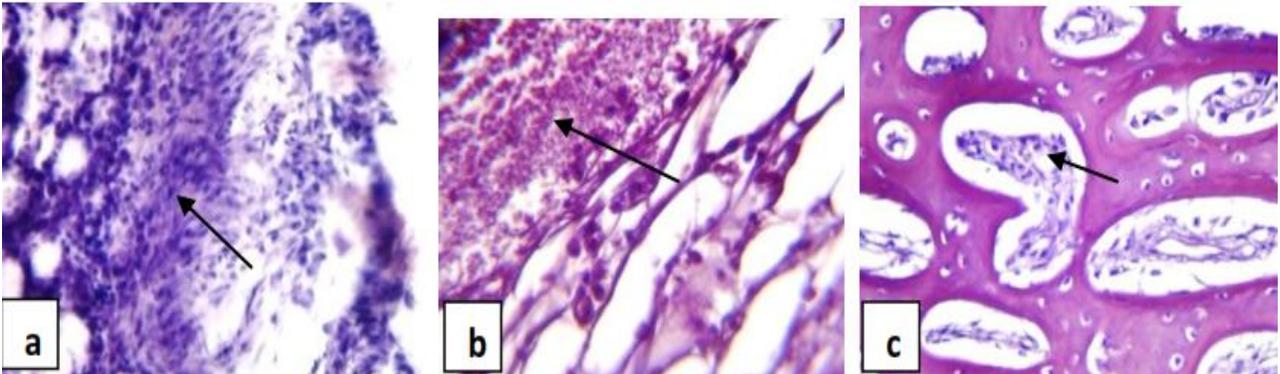
FIGURE 4: Tibia of non-immunized group at 15 day post challenge (H & E stain) (a) infiltration of neutrophils and F.C.T (arrow) in the medullary cavity (×20), (b) neutrophils infiltration, coagulative necrosis (arrow) and loss of differential staining of marrow elements with circular space (pooled lipid) (×20), (c) neutrophils in dilated Haversian canals (arrow) (×20).

At 30 day post infection the bone marrow showed congestion of blood vessels, hemorrhage, osteoclasts proliferation, abscess, necrosis and F.C.T. proliferation, also proliferation of F.C.T. in the medullary cavity of trabecular bone which revealed erosion and necrosis, The lesion extended from marrow to the necrotic cortical bone forming a pocket filled with neutrophils and MNCs, the necrotic bone tissue characterized by loss of differential stain and pyknotic of osteocytes and empty or dialated lacunae.

The Haversian canals were filled with neutrophils and/or osteoclasts, while the periosteum become irregular. However, the lesion extended to the subchondral bone and to the articular cartilage which showed multiple areas of erosion and necrosis, the necrotic cartilage loss its differential stain and the chondrocytes are pyknotic or completely disappeared and invaded by inflammatory cells mainly neutrophils (Fig. 5).
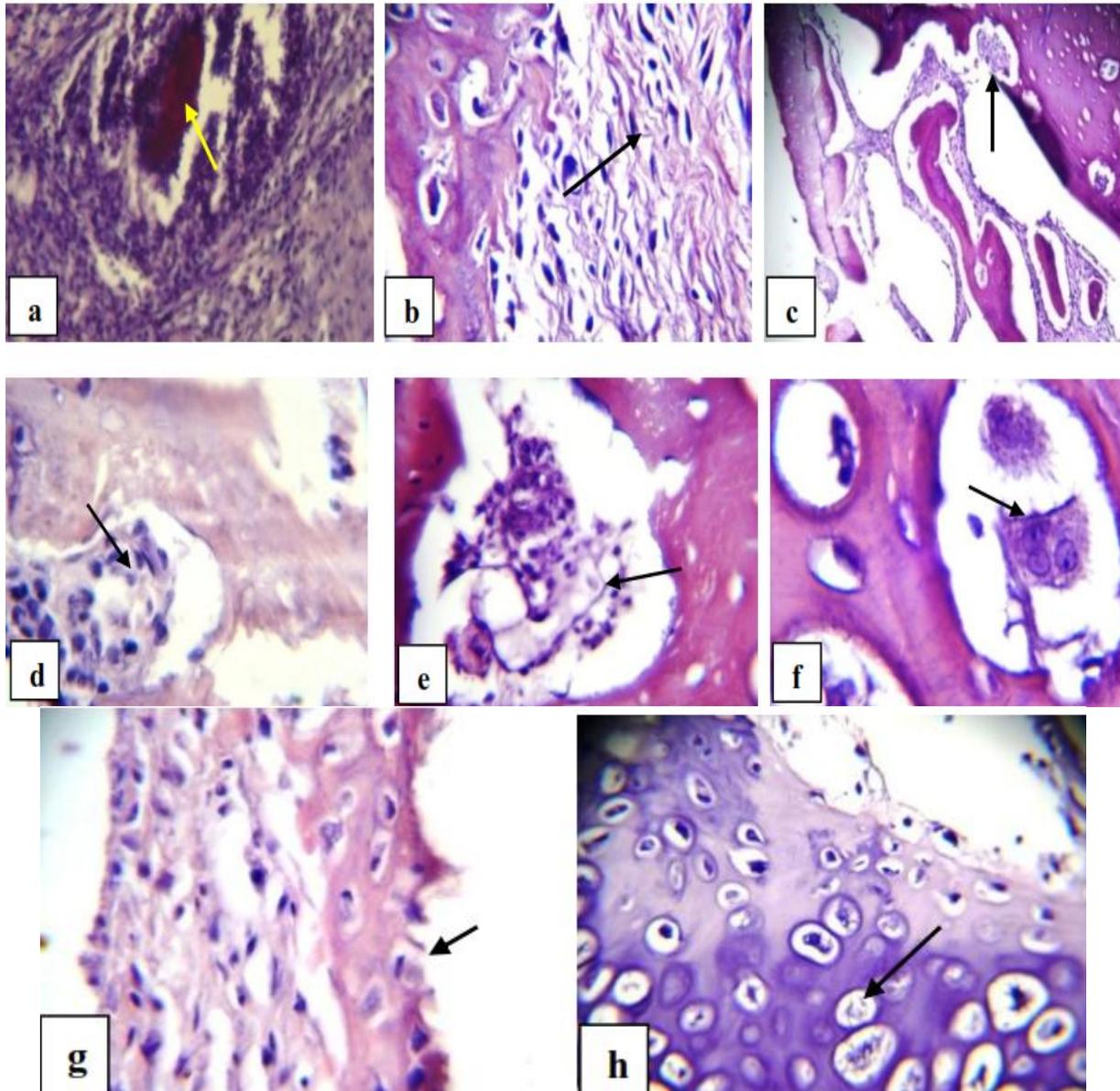
FIGURE 5: Tibia of non-immunized group at 30 day post challenge (H & E stain): (a) abscess and necrosis (arrow) were seen in the bone marrow (×20), (b) F.C.T. proliferation in the bone marrow (arrow) (×20), (c) proliferation of fibrous connective tissue in the medullary cavity of trabecular bone which revealed erosion and necrosis (arrow) (×10), (d) a pocket in the necrotic cortical bone filled with neutrophils and MNCs (arrow) (×40), (e) Haversian canal filled with neutrophils (arrow) (×40), (f) Haversian canals were filled with osteoclasts (arrow) (×40), (g) erosion of periosteum (arrow) (×20) (h) necrotic articular cartilage loss its differential stain and chondrocytes are pyknotic or completely disappeared (arrow) (×40).

The lesion in the immunized group was less severe, at day 5 post challenge the lesion was confined to bone marrow which show moderate infiltration of neutrophils and osteoclast proliferation (Fig.6).
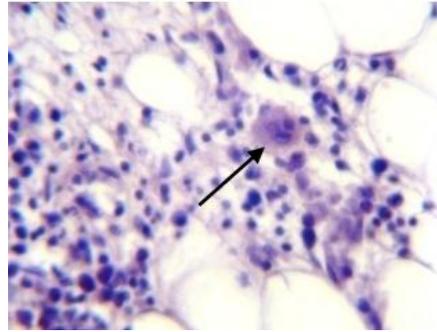
FIGURE 6: Tibia of immunized rabbit 5 dayt post challenge (H & E stain), osteocalsts proliferation (arrow) (×40)

At 15 days post infection the bone marrow showed congestion of blood vessels, neutrophils and few macrophages surrounding a fragment of necrotic bone. There is vascular F.C.T. and proliferation of osteoclasts in the bone marrow, osteoclasts absorb the dead bone forming a hallow shape. The trabecular bones were lining by active osteoblasts in addition to few inflammatory cells in the Haversian canals. However, the inflammatory reaction did not extend to the subchondral bone or articular cartilage (Fig. 7).
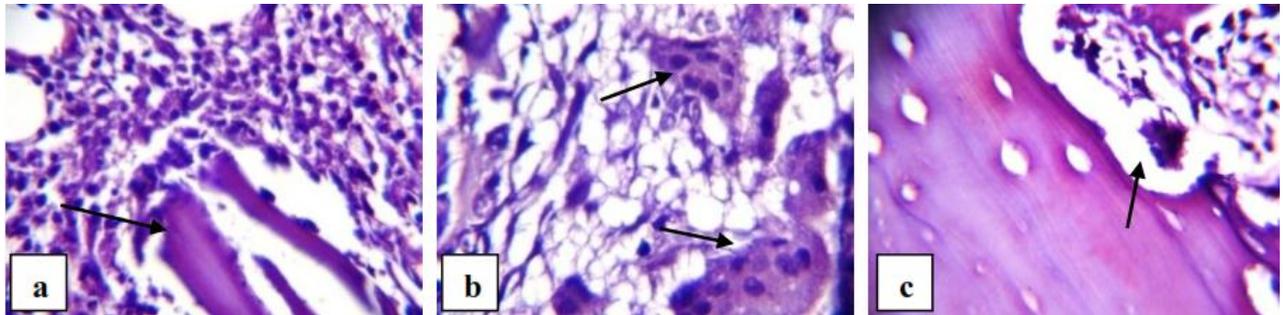
FIGURE 7: Tibia of immunized group at 15 day post challenge (H & E stain) (a) neutrophils and few macrophages surrounding a fragment of necrotic bone (arrow) (×20), (b) proliferation of osteoclasts the bone marrow (arrow) (×40), (c) osteoclasts absorb the dead bone forming a hallow (arrow) (×20).

At 30 days post infection immunized group showed vascular F.C.T. infiltrated with round macrophages in the bone marrow surrounded the reactive new bone, also neutrophils infiltration in bone marrow and large number of osteoclasts in a hollow shape lacuna in the endosteum. Active osteoblasts lining the surface of trabecular bone and infiltration of MNCs and F.C.T. in medullary cavity of trabecular bone, also woven bone formation extended from the endosteum to bone marrow with wide space between the trabiculae, the space filled with cells that arranged in irregular manner. The cortical bone showed dilated Haversian canal and inflammatory cells in there lumen (Fig. 8).
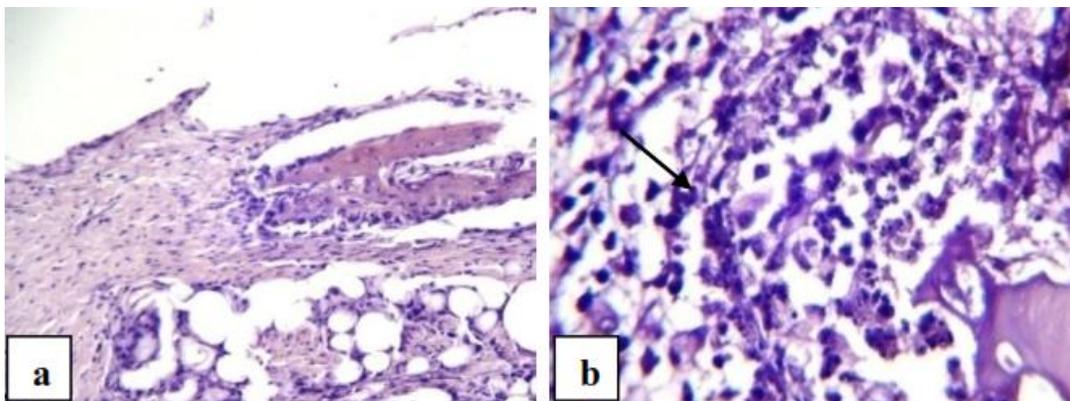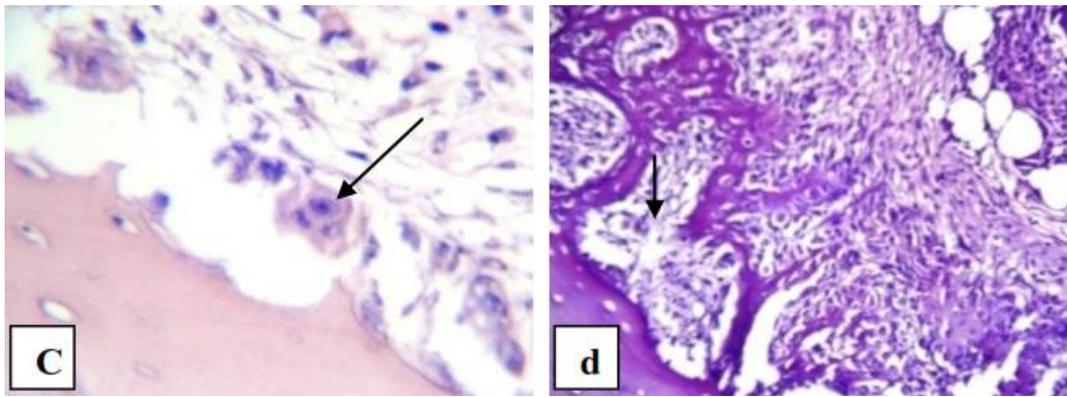
FIGURE 8: Tibia of immunized group at 30 day post challenge (H & E stain) (a) vascular F.C.T. infiltrated with round macrophages in the bone marrow (×10), (b) neutrophils infiltration (arrow) in bone marrow (×20), (c) osteoclasts in a hollow shape lacuna (arrow) (×40), (d) woven bone formation with wide cavity filled with cells arranged in irregular manner (arrow) (×10).

Hypercellularitey of bone marrow may be due to neutrophils infiltration which is also seen in Haversian canals and this refers to the acute phase of the infection since neutrophils is the major cells type responsible for clearance of *P. aeruginosa* (Sadikot *et al.*, 2005), Necrosis of neutrophils and hemopoietic component may be contributed to the virulence factors of *P. aeruginosa* such as exotoxin A which had a local necrotizing activity aid in the colonization of the bacteria (Todar, 2009). In addition *P. aeruginosa*, protect itself from host defense mechanism by production of biofilm (Bjarnsholt *et al*., 2008), the presence of neutrophils is detected by biofilm cells of *P. aeruginosa* which direct this information to their fellow bacteria through the quorum sensing (QS) signaling system (Alhede *et al*., 2009), the bacteria upregulating synthesis of a number of QS-controlled virulence determinants including rhamnolipids which causes necrosis of neutrophils (Jensen *et al.*, 2007), invasion and necrosis of tissue due to degeneration of lipids and lecithin (Westman *et al*., 2010), increase resistant of biofilm to phagocytic cells (Morici *et al*., 2007), and distortion of macrophages and inhibited their ability to bind and /or ingest preopsonized *P. aeruginosa*m (Westman *et al*., 2010). The presence of neutrophils in the Haversian and Volkmann's canals indicate the perforation of acute inflammation through the cortical bone and under the periosteum, which is elevated by the inflammatory process (Hsien and Yung-Chien, 2012).

Alkaline protease and LasB may cause severe damage and necrosis of bone tissue by reducing the phagocytic activity against *P. aeruginosa* and the ability of these virulence factors to degraded IL-2 which impair lymphocyte function (Kharazmi *et al*., 1986), also *P. aeruginosa* can induced both apoptotic and necrotic cell death in macrophages, which may contribute as a mechanism of immune suppression leading to exacerbation of inflammation (Dockrell and Whyte, 2006). In addition ExoS and ExoT can cause rearrangement of the actin cytoskeleton and inhibition of phagocytosis (Barbieri and Sun, 2004).

The hemorrhage in the bone marrow may caused by bacterial protease IV which cleaved fibrinogen, this dysfunction will lead to hemorrhage which is characteristic of *P. aeruginosa* infection (Elliott and Cohen 1986).

Osteoclasts proliferation suggested that LPS can act as a potent stimulator of bone loss by causing an increase in the number of osteoclasts in mice, also TLR activation can enhance osteoblast-mediated osteoclast differentiation by inducing RANKL and TNF- on osteoblasts (Hayashi *et al*., 2003)**.**

The extension of the lesion to the articular joint at 30 days post inoculation was in compatible with a previous study (Al-awadi and Alwan, 2014) whom expleained the destructive effects of *P. aeruginosa* on articular cartilage can contribute particular tropism of *P. aeruginosa* for fibrocartilagenous tissue (Todor 2009), also the inflammatory response increased the intraosseous pressure, which impairs blood flow and leads to ischemic necrosis (sequestrum) Fritz and McDonald 2008).

Immunized rabbits showed less severe inflammatory reaction in the medullary cavity and Haversian canals and this indicated that OMAgs stimulated immune response that destroyed most of the microorganism at the site of inoculation and these result was confirmed by immunological tests (DTH, IFN- and IgG), that's means both cellular and humoral immune responses have synergistic action against *P. aeruginosa* infection, this investigation was supported the ideas that the phagocytic cells (Aderem and Ulevitch, 2000), complement (Hauser *et al*., 2002) and immunoglobulines (Moser *et al.*, 1999) plays an essential role in protection of the host against *P. aeruginosa* infection. The current results suggested that OMAgs may lead to the production of antibodies that prevented bacterial adherence and neutralized cytotoxic activity of bacterial toxins. These facts may explained the localization of the lesion in the immunized group to the bone marrow and nearby cortical bone.

In addition, the present of active osteoblasts lining the trabecular bone be due to the activation of the osteoblast by Th-1 cytokines. These evidence were in agreement with Lorenzo *et al.*, (2008) and Lorenzo *et al*., (2011) who reported that multiple cytokines, chemokines and growth factors of the immune cells such as T and B cells, fibroblasts, and macrophages directly or indirectly regulate osteoblast and osteoclast activity by producing or influencing the production of the RANKL/RANK

pathway, TNF- and IFN- . also Takayanagi, (2009) noticed that the osteoimmunology based on the functional interdependence between the immune system and bone at the anatomical, vascular, cellular, and molecular levels.

CONCLUSION
P. aeruginosa produce osteomyelitis and the infection extend to produce arthritis, OMAgs of *P. aeruginosa* was a potent immune stimulator that enhanced both cellular (DTH and IFN-) and humoral (IgG) immunity against *P. aeruginosa* and limiting the lesion in the bone to a great extent.

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